The Msi Family of RNA-Binding Proteins Function Redundantly as Intestinal Oncoproteins

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SUMMARY

Members of the Msi family of RNA-binding proteins have recently emerged as potent oncoproteins in a range of malignancies. Msi2 is highly expressed in hematopoietic cancers, where it is required for disease maintenance. In contrast to the hematopoietic system, colorectal cancers can express both Msi family members, Msi1 and Msi2. Here, we demonstrate that, in the intestinal epithelium, Msi1 and Msi2 have analogous oncogenic effects. Further, comparison of Msi1/2-induced gene expression programs and transcriptome-wide analyses of Msi1/2-RNA-binding targets reveal significant functional overlap, including induction of the PDK-Akt-mTORC1 axis. Ultimately, we demonstrate that concomitant loss of function of both Msi family members is sufficient to abrogate the growth of human colorectal cancer cells, and Msi gene deletion inhibits tumorigenesis in several mouse models of intestinal cancer. Our findings demonstrate that Msi1 and Msi2 act as functionally redundant oncoproteins required for the ontogeny of intestinal cancers.

INTRODUCTION

Mammalian orthologs of the Drosophila melanogaster Musashi RNA-binding protein include Msi1/MSI1 and Msi2/MSI2. Drosophila Musashi governs asymmetric cell fate determination in neuroblasts through translational suppression of mRNAs encoding a lineage determinant (Nakamura et al., 1994; Okabe et al., 2001). A similar role for Msi2 in regulating asymmetric fate determination has been proposed based on analysis of asymmetric partitioning of the Msi RNA-binding target Numb in hematopoietic stem cells with Msi2 gain or loss of function (Kharas et al., 2010; Park et al., 2014). Besides a potential role in governing asymmetric cell division, Msi proteins act as potent oncoproteins in a number of cancers. In particular, Msi2/MSI2 is a cooperative oncoprotein in hematopoietic malignancies, where it sustains a cancer stem cell self-renewal program through interaction with a number of mRNA-binding targets (Itt et al., 2010; Kharas et al., 2010; Park et al., 2014, 2015). Whereas significant progress has been made in understanding the contribution of Msi2 to hematopoietic malignancies, very little is known about the functional contribution of Msi proteins to oncogenic transformation in other human malignancies and murine tumor models.

In the hematopoietic system, Msi2 is the only Msi family member expressed and its expression is largely restricted to the hematopoietic stem cell compartment. In contrast, Msi1 and Msi2 are coexpressed in the putative stem cell compartments of a variety of other tissues including the hair follicle (Sugiyama-Nakaigiri et al., 2006), mammary gland (Clarke et al., 2003; Katz et al., 2014; Wang et al., 2008), germ cells (Sutherland et al., 2014), intestinal epithelium (Kayahara et al., 2003; Li et al., 2014; Potten et al., 2003; Wang et al., 2015), and neural epithelium (Sakakibara et al., 2002). The observation that both Msi1 and Msi2 are coexpressed in these tissues, coupled with an absence of phenotype upon genetic ablation of either Msi1 or
MsI2 (with the exception of compromised brain ventricle formation in MsI1−/− mice), and sequence homology between MsI1 and MsI2 strongly suggests that functional redundancy exists between MsI family members. This notion is supported by findings where knockdown of MsI2 in ex vivo cultures of MsI1-null neurospheres inhibits self-renewal (Sakakibara et al., 2002).

Here, we compare the similarities between the oncogenic properties of MsI1 and MsI2 in the intestinal epithelium. Published studies have observed MsI1 expression in both stem cells of the intestinal crypts and human colorectal cancers (CRCs), and several studies suggest that MsI1 has mitogenic activity driven by potentiation of the canonical Wnt- and/or Notch-signaling pathways (Cambuli et al., 2015; Rezza et al., 2010; Spears and Neufeld, 2011; Sureban et al., 2008). Like MsI1, MsI2 is also expressed in the stem cell compartment of intestinal crypts and is broadly overexpressed in CRC, and MsI2 inhibition in SW48 and HT29 CRC cell lines has antiproliferative effects both in vitro and in murine xenografts (Wang et al., 2015). These findings suggest that MsI1 and MsI2 may have overlapping roles in promoting transformation of the intestinal epithelium; however, in vivo gain-of-function studies demonstrated that MsI2, unlike MsI1, does not potentiate canonical Wnt signaling and has little to no effect on activity of the Notch pathway as was reported for MSI1 in CRC cell lines (Wang et al., 2015). Thus, there is uncertainty as to whether MsI1 has oncogenic functions in the intestinal epithelium in vivo and whether MsI1 and MsI2 might function redundantly in promoting intestinal transformation.

Here, we demonstrate that acute, in vivo MsI1 gain of function phenocopies that of MsI2, including expansion of intestinal crypt base columnar stem cells, blocked differentiation, upregulation of an APC-loss gene expression signature, and activation of the mTORC1 complex, all in a Wnt-independent manner. Comparison of transcriptome profiles derived from either MsI1 or MsI2 gain of function indicates that these families are capable of activating similar gene expression programs, and comparison of transcriptome-wide, in vivo, MsI1- and MsI2-RNA-binding analyses reveals a similar repertoire of binding targets that function in analogous pathways. Ultimately, MsI1/2 loss-of-function experiments demonstrate that the activity of these RNA-binding proteins is required for the initiation and maintenance of intestinal cancers.

RESULTS

MsI1 Is Expressed in Intestinal Cancers, and Its Forced Expression Transforms the Intestinal Epithelium

Several studies suggest that MSI1 and MSI2 are broadly expressed in CRCs (Levin et al., 2010; Li et al., 2011; Wang et al., 2015), although correlation of MSI expression with stage or grade is less clear. We sought to confirm MSI1 expression in gastrointestinal cancers and observed both over- and underexpression relative to controls in a number of these malignancies (Figures 1A, 1B, 1S1, and 1S2). Interestingly, matched pairs of colorectal adenocarcinomas and normal adjacent tissue revealed MSI1 expression to be more variable than that of MSI2, with MSI1 overexpression observed in around half of these malignancies in comparison to the ubiquitous overexpression of MSI2 (Figure 1A; Wang et al., 2015). We confirmed prior reports of MSI1 expression in murine crypt base columnar intestinal stem cells (CBCs) and its upregulation in early adenomas resulting from loss of heterozygosity of the APC tumor suppressor in the APCmin/+ mouse model (Potten et al., 2003; Figures 1C and 1D). Given that MSI2 is broadly expressed in CRC and is also upregulated upon APC loss, we generated a targeted, single-copy, doxycycline-inducible MSI1 gain-of-function mouse model (TRE-Msi1) using genetic loci identical to those used to assess the consequences of MSI2 gain of function (Wang et al., 2015). This enables direct comparison between the consequences of MsI1 and MsI2 activation (Figures 1E, 1S1C, and 1S1D). Doxycycline (Dox) administration resulted in broad induction of MsI1 throughout the intestinal epithelium, but not in the underlying stromal mesenchyme or lacteals (Figure 1S1E). Control mice (R26-M2rtTA + Dox) exhibited no differences in MsI1 expression patterns relative to wild-type (Figure 1S1E).

MsI1 induction resulted in an expansion of the crypt proliferative zone and decreased presence of differentiated cells (the exception being persistence of Paneth cells, possibly due to their long lifespan and stable positioning at the crypt base; Figures 1A, 1B, 1S1, and 1S2). These findings suggest that MsI1 has mitogenic activity that is shared with MsI2, consistent with the notion that these RNA-binding proteins are functionally redundant in promoting intestinal epithelial transformation.

Figure 1. MSI1 Is Expressed in Colorectal Cancers and Is Sufficient to Transform the Intestinal Epithelium

(A) MSI1 expression in matched tumor/control sample pairs from TCGA colorectal adenocarcinoma (COAD) RNA-seq (total of 26 patients). The distribution of MSI1 fold changes in tumor/control pairs for 26 individuals is plotted in red (intra-individual comparison). The distribution of MSI1 fold changes between control/comparison comparisons for 26 pairs of healthy individuals is plotted in red (intra-individual comparison). The distribution of MSI1 fold changes between control/comparison comparisons for 26 pairs of healthy individuals is plotted in red (intra-individual comparison).

(B) Immunohistochemical staining for MSI1 in graded human colorectal cancer sections (scale = 100 μm).

(C) Immunofluorescence staining of MSI1 (red) in cells of the intestinal crypt stained for the crypt base columnar stem cell marker Lgr5 in Lgr5-eGFP-IRESCreER knockin mice (green; scale = 50 μm).

(D) Immunofluorescence staining for β-catenin and MSI1 in an adenoma resulting from APC LOH in the APCmin/+ mouse and normal vili adjacent to the adenoma (scale = 100 μm).

(E) Design of doxycycline (Dox)-inducible MSI1 knockin mouse harboring a modified reverse tetracycline transactivator (M2rtTA) at the ROSA26 locus and the MSI1 cDNA under control of the tetracycline-responsive element-minimal CMV promoter (TRE/TetOP) targeted to safe-haven chromatin downstream of the Col1a1 locus.

(F) Immunofluorescence staining for Ki-67 marking proliferative cells in the intestinal crypts of control (M2rtTA) and TRE-MSI1 mice 48 hr after Dox induction.

(G) Alcian blue staining for goblet cell mucin.

(H) Immunofluorescence staining for the enteroendocrine marker chromogranin A (scale in F–H = 100 μm).

(I) Histological sections showing extension of crypt height and increased crypt fission in TRE-MSI1 mice (quantified at right; n = 3 mice; ***p<0.0005; Student’s t test).

(J) A crypt undergoing fission in Lgr5-eGFP-IRESCreER knockin mice costained for MsI1 (red) and GFP (green; scale = 50 μm).

See also Figure S1.
Figure 2. Msi1 Induction Expands the Progenitor Cell Compartment and Drives APC Loss and RNA Metabolism Gene Expression Programs

(A) Msi1 induction in TRE-Msi1::Lgr5-eGFP-CreER mice results in an upward expansion of Lgr5-eGFP+ cells and an increase in the absolute frequency of Lgr5-eGFP+ cells, quantified by flow cytometry (right; n = 3 mice per group; *p < 0.05; Student’s t test; scale = 50 μm).

(B) TRE-Msi1 epithelium transformed by Dox induction for 48 hr revert to a phenotypically normal state persisting 2 months after Dox withdrawal (scale = 100 μm).

(C and D) In vitro culture of intestinal organoids derived from TRE-Msi1::Lgr5-eGFP-CreER crypts followed by Dox induction in vitro. Crypt bud length is quantified in (D) (**p < 0.0005; Student’s t test).

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1F–1H and S1F–S1I). This caused TRE-$\text{Msi1}$ mice to become dehydrated, requiring euthanasia after approximately 3 days of Dox exposure. Msi1 induction also increased crypt fission, and endogenous Msi1 colocalized with Lgr5+ CBCs at sites of crypt fission in wild-type mice (Figures 1I and 1J). We next examined the effects of Msi1 induction on CBCs in TRE-Msi1::Lgr5-eGFP-CreER mice and observed an upward expansion of the stem cell zone and a significant increase in the frequency of Lgr5-eGFP+ CBCs (Barker et al., 2007; Figure 2A). Dox withdrawal resulted in a reversion of the epithelium to the wild-type state, which was maintained for several months, indicating that, upon Msi downregulation, crypt stem cells return to a niche-dependent homeostatic state (Figure 2B). Further, Msi1 induction in ex vivo cultures of Lgr5-eGFP+ crypts resulted in increased growth of crypt buds, indicating that the phenotype is epithelial cell-autonomous (Figures 2C and 2D).

All of the phenotypes resulting from Msi1 induction are consistent with those observed in response to Msi2 induction and are also consistent with acute loss of APC (excepting the stable positioning of Paneth cells, which become mislocalized upon APC loss; Sansom et al., 2004; Wasan et al., 1998). Thus, Msi1 induction in vivo phenocopies that of Msi2 in otherwise genetically identical mouse models.

$\text{Msi1 and Msi2 Drive Common Gene Expression}$

$\text{Programs and Interact with Common Target Transcripts}$

We next analyzed changes to the transcriptome resulting from Msi1 induction (Table S1). Consistent with our phenotypic observations, unbiased gene set enrichment analysis (GSEA) (Subramanian et al., 2005) demonstrates that the APC-loss gene signature is among the most highly enriched upon Msi1 induction (Figures 2E and 2F; Table S2). We also observed enrichment of gene sets related to mRNA processing and translation along with an inverse correlation between the Msi1-induced gene expression program and expression profiles induced by the mTORC1 inhibitor rapamycin (Figures 2G and 2H). All of these gene signatures were similarly enriched in intestinal epithelium overexpressing Msi2 (Wang et al., 2015), indicating that the analogous phenotypes resulting from Msi1 or Msi2 induction are driven by analogous gene expression programs. We therefore directly compared Msi1- and Msi2-induced transcriptome profiles and observed that 72% of gene expression changes resulting from Msi1 induction also occurred upon Msi2 induction (Figure 3A). Gene ontology (GO) and pathway analysis found commonly upregulated programs involved in ribosome biogenesis, signal transduction, and ErbB signaling, among others (Table S3). Commonly downregulated programs were broadly related to oxidative phosphorylation and mitochondrial activity.

Given that Msi1/2 are RNA-binding proteins that act on translation (Battelli et al., 2006; Katz et al., 2014; Kawahara et al., 2008; Okano et al., 2002), we performed in vivo transcriptome-wide RNA-binding analysis (CLIP-seq) for both endogenous and induced Msi1 in the intestinal epithelium to identify direct targets. Msi1 bound 2,371 transcripts in wild-type crypts, primarily in 3′ UTRs and coding sequences (Figure 3B; Table S4). Upon Dox administration, ectopic Msi1 became increasingly associated with intronic sequences, and 91% of transcripts newly bound upon Msi1 induction (i.e., those transcripts associated only with ectopic Msi1) were bound in introns (Figures 3B and S2A). In total, 93% of transcripts bound by Msi1 in wild-type crypts were also bound in TRE-Msi1 epithelium (Figure 3C). These binding patterns were consistent with those previously described for Msi2.

Motif analysis of CLIP targets indicated that Msi1 binds distinct sequences in introns and coding sequences in comparison to 3′ UTRs, and although Msi1-binding motifs were A-U rich, the previously described Msi1 motif discovered by a SELEX-based approach ((A/U)U(A/U)AGU; Imai et al., 2001) was not among the most significant (Figure 3D). It was, however, significant in the data set and was located in the center of sequence reads containing it, demonstrating that it is a bona fide motif in vivo (Figures 3D and 3E). Msi1 binding showed no preference for highly abundant transcripts, and globally, transcripts newly bound upon Msi1 induction exhibited no change in their expression levels (Figures S2B and S2C). Sequence-specific Msi1-RNA interactions were confirmed using in vitro binding assays incubating recombinant human Msi1 with RNA oligos containing the consensus motifs identified by CLIP (Figure S2D).

The large number of intronic binding sites led us to further investigate Msi1-intronic interaction. Nuclear/cytoplasmic fractionation of wild-type crypts and human CRC cells indicates that the majority of Msi1 is cytoplasmic, and binding site conservation analysis demonstrates that, whereas all binding sites for endogenous Msi1 and the UTR and coding sequence sites for ectopic Msi1 are evolutionarily conserved, intronic binding sites of ectopic Msi1 are not (Figures 3F and S2E). These data suggest that intronic binding events for ectopic Msi1 may be largely promiscuous. Consistent with this, analysis of the location of Msi1-binding sites across introns revealed a preference for interaction of Msi1 with the 5′ intron terminus in wild-type crypts, and this preference was largely attenuated for ectopic Msi1 (Figure S2F). This also suggests that some Msi1-intron interactions may be involved in alternative splicing (Katz et al., 2014; Uren et al., 2015). We therefore asked how Msi1 binding was related to exon inclusion/exclusion. Interestingly, Msi1 targets were more likely to contain exons that exhibited increased inclusion upon ectopic Msi1 induction than transcripts not bound by Msi1, further suggesting that Msi1 activity may be associated with alternative exon inclusion (Figure S3A).

See also Tables S1 and S2.

(E) Heatmap and hierarchical clustering of transcriptome profiles performed on the intestinal epithelium of three control (M2rtTA) and three TRE-Msi1 mice treated with Dox for 24 hr. (F–H) Gene set enrichment analysis (GSEA) of the TRE-Msi1 transcriptome identifies activation of genes induced by acute APC deletion in the intestinal epithelium (APC loss up) and suppression of genes downregulated after APC deletion (APC loss down; F), along with an anti-correlation between the Msi1-induced transcriptome profile and the Peng_Rapamycin UP/DOWN gene sets (G) and an enrichment of expression of mRNA processing, ribosomal, and translation factors upon Msi1 induction (H). FDR, false discovery rate.
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**Msi1 and Msi2**

Similar binding patterns were observed for ectopically induced an unrelated RNA-binding protein Lin28b (Madison et al., 2013). Consistent with the GSEA and GO analyses, Ingenuity Pathway Analysis identified pathways involved in cellular respiration, cancer, and Pten-PI3K-AKT-mTORC1 signaling as being the most significantly represented across the CLIP data sets (Figure S3B).

We next sought to determine the overlap in the RNA-binding activities of Msi1 and Msi2 and observed that 69% and 68% of transcripts bound by wild-type Msi1 in their 3’ UTR and coding sequences, respectively, were similarly bound by Msi2 (Figure 3H). Similar binding patterns were observed for ectopically induced Msi1 and Msi2 (Figure S3C). Both Msi1 and Msi2 targets functioned in analogous pathways, including RNA metabolism, protein localization, and cellular respiration (Figures 3G and S3B; Tables S5 and S6). Further, overlap of our murine in vivo CLIP data sets with published RNA-binding data for MSI1 in human transformed cell lines in vitro (de Sousa Abreu et al., 2009; Uren et al., 2015; Vo et al., 2012) reveals a significant overlap despite the vast differences in cellular identity (Figure S4A). These analyses indicate that Msi1/Msi2 act on a common set of target genes, accounting for the identical phenotype between Msi1 and Msi2 gain of function. Given their highly similar RNA-binding activities, we tested for Msi1-Msi2 protein interaction but failed to detect any by co-immunoprecipitation, suggesting that either Msi1 or Msi2 are sufficient to act upon target transcripts (Figure S4B).

The transcripts encoding p21 and Numb are among the best-characterized Msi1 targets (Sattelli et al., 2006; Imai et al., 2001), and we previously observed Msi2 binding to these transcripts in the intestinal epithelium (Wang et al., 2015). Similarly, these transcripts were bound by ectopically induced Msi1 (Figure S4C). Msi1 also bound the transcripts encoding the well-established intestinal tumor suppressors Lrig1 and BmpR1 (He et al., 2004; Powell et al., 2012), as we previously observed for Msi2 (Figure 4A). Interestingly, Lrig1 is a negative regulator of ErbB signaling, and we observed upregulation of the ErbB pathway downstream of both Msi1 and Msi2 (Table S3).

### Msi1 Does Not Potentiate Transcriptional Activity of the Canonical Wnt Pathway

Msi1 has been reported to bind to and inhibit the function of APC, a negative regulator of the transcriptional effector or canonical Wnt signaling, β-catenin (Spears and Neufeld, 2011), and in vitro studies have posited a role for Msi1 in potentiating canonical Wnt target gene expression (Rezza et al., 2010). Indeed, we observed binding of the APC transcript; however, we also observed stronger binding of Msi1 to the Ctnnb1 transcript encoding β-catenin (Figure 4A). We confirmed the preference of endogenous human Msi1 for CTNNB1 versus APC using CLIP-qRT-PCR, and this binding preference was maintained upon Wnt pathway stimulation with the GSK3β-inhibitor CHIR 99021 (Figure 4B). 3’ UTR luciferase reporter assays confirmed that Msi1 repress CTNB1 translation, albeit moderately (Figure 4C). Similarly, activity of the TOPFlash dualimerized β-CATENIN/TCF reporter was attenuated by MSI1 activity (Figure 4D). To determine the effects of Msi1 activity on the canonical Wnt pathway in vivo, we examined APC and β-catenin protein in control and TRE-Msi1 epithelium and observed no appreciable differences (Figures 4E–4G). Further, analysis of the expression of direct β-catenin target genes in TRE-Msi1 epithelium showed no significant changes upon Msi1 induction (Figure 4H). Thus Msi1, like Msi2, does not potentiate canonical Wnt signaling in vivo.

### Msi1 Activates the PDK-Akt-mTORC1 Axis

One of the major oncogenic pathways downstream of Msi2 is mTORC1, which becomes activated upon Msi2 binding to its upstream inhibitor Pten (Wang et al., 2015). Msi1 also bound to the 3’ UTR of the Pten mRNA, decreasing Pten protein levels (Figures 5A and 5B). Further, MSI1 knockdown in human CRC cells led to increased PTEN activity and decreased PIP3 levels (Figures 5C and SSA-SSC). Downstream of Pten, Msi1 induction activated the PDK-Akt-mTORC1 axis (Figures 5B and 5D), with increased phosphorylation of AKT at T308 by PDK1 (PDPK1). AKT phosphorylation at S473 by mTORC2, however, showed only a minor increase, indicating that mTORC1 activation is due to increased AKT activity via activation of AKT by the PI3K pathway. Consistent with this notion, we observed an increase in the activating PDK1 autophosphorylation event at S241 (Figure 5D) and increased phosphorylation of the AKT target c-RAF (S259), further supporting the activation of AKT downstream of Msi1. Downstream of mTORC1, there was strong inactivating phosphorylation of the translational inhibitor 4EBP1, resulting in translation repression of target genes.

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**Figure 3. Msi1 and Msi2 Have Overlapping RNA-Binding Targets**

- **A.** Venn diagram showing the degree of overlap in gene expression changes driven by Msi1 versus Msi2 induction in transcriptome profiles of the TRE-Msi1 and TRE-Msi2 intestinal epithelium.
- **B.** Distribution of Msi1-RNA binding events for endogenous Msi1 in wild-type crypts (left) and induced Msi1 in TRE-Msi1 intestinal epithelium (right).
- **C.** Venn diagram showing the degree of overlap in Msi1 RNA-binding targets wild-type crypts and in mutant mouse intestinal epithelium.
- **D.** Msi1-binding motif identification and distribution in wild-type intestinal crypts. The fifth motif represents the motif previously identified by selex in vitro.
- **E.** Position of the canonical Msi1 recognition motif previously identified in vitro within CLIP-seq reads containing that motif.
- **F.** PhastCons analysis of conservation of Msi1-binding sites in the indicated regions of Msi1 target transcripts. Error bars represent 95% confidence intervals; “p < 0.05; “p < 0.005; “p < 0.0005.
- **G.** Gene ontology analyses for biological processes and molecular functions that are significantly enriched in Msi1/TRE-Msi1 or Msi2/TRE-Msi2 CLIP data sets, as well as for gene sets common to both wild-type or ectopic Msi1 and Msi2, or targets unique to Msi1/TRE-Msi1 or Msi2/TRE-Msi2, as well as targets bound by an unrelated RNA-binding protein Lin28b.
- **H.** Venn diagrams showing overlap in transcripts bound by endogenous Msi1 and Msi2 in wild-type crypts.

See also Figures S2–S4 and Tables S3, S4, S5, and S6.
Figure 4. Effects of Msi1 on Wnt Pathway Activity

(A) CLIP-seq tracks showing endogenous (WT) and ectopically induced Msi1-binding target transcripts.

(B) CLIP-qRT-PCR analysis of endogenous MSI1 binding to 3' UTRs of APC and CTNNB1 (β-CATENIN) in HEK293 cells in the absence (Ctrl) or presence of the GSK3β inhibitor CHIR99021 (CHIR) (n = 3). ***p < 0.0005; Student’s t test.

(C) Luciferase reporter assays in HEK293 cells upon lentiviral shRNA knockdown of MSI1 (using pSico) or MSI1 overexpression (using pcDNA), shown for constructs containing the CTNNB1 and NUMB 3' UTRs (n = 3; *p < 0.05; ***p < 0.0005; Student’s t test).

(legend continued on next page)
activation of the translational initiation factor eIF4E (Figure 5D). Examination of the levels and spatial distribution S6 phosphorylation (as an ultimate readout of S6 kinase activity downstream of mTORC1) revealed a dramatic induction and expansion throughout the entire epithelium upon Msi1 induction (Figures 5B and 5E). Thus, activation of the PDK1-AKT axis downstream of Pten by Msi1 contributes to the observed increase in mTORC1 activity in the intestinal epithelium. To confirm the functional importance of mTORC1 activity for the Msi1-driven phenotype, we pre-treated TRE-Msi1 mice with rapamycin prior to inducing Msi1 with Dox. Rapamycin treatment blocked crypt fission, crypt height expansion, and hyperproliferation in the presence of ectopic Msi1 activity, confirming the functional importance of the mTORC1 complex downstream of Msi1 (Figures 5F–5H).

**Msi Activity Is Required for Intestinal Tumorigenesis**

The data thus far establish that Msi1 activity is sufficient to transform the intestinal epithelium in a manner analogous to Msi2 and that these two RNA-binding proteins act on a similar set of target transcripts and affect analogous downstream pathways. If Msi1 and/or Msi2 act as oncoproteins in intestinal cancers, our data predict that the expression of one or the other would be sufficient to drive malignancy. We therefore assessed expression of Msi1 and Msi2 in a panel of human CRC cells and found that, whereas Msi2 was consistently expressed across all cell lines, Msi1 was coexpressed only in a subset and was frequently expressed at levels lower than those observed in normal human colon (Figure 6A). This pattern is precisely what we would predict from TCGA data analysis of paired tumor/adjacent normal tissue (Figure 1A; Wang et al., 2015). We thus inhibited Msi1 alone or in combination with Msi2 and/or β-Catenin in several CRC cell lines. Msi1 inhibition alone had an antiproliferative effect in some lines, but not others (Figure 6B), and this effect could not be predicted by levels of Msi1 expression relative to Msi2. Concomitant inhibition of both Msi1/Msi2 significantly inhibited growth of all cell lines analyzed, and additional inhibition of β-Catenin blocked tumor cell growth more effectively than β-Catenin or Msi inhibition alone (Figure 6B). Immunoblotting for Msi1 and nuclear (transcriptionally active) β-Catenin indicates that Msi and β-Catenin act in parallel pathways, as β-Catenin knockdown has no major effect on Msi1 protein levels and vice versa (Figures 6C and 6A). We further confirmed that Msi1 inhibition abrogated the growth of tumor xenografts from RKO and HCT116 cells and that addition of β-Catenin inhibition synergized with Msi inhibition to completely block tumor growth (Figures 7A–7D, S6B, and S6C).

These findings suggest that Msi activity is required for the maintenance of CRC growth. To test this in a more physiologically relevant setting and to address the importance for Msi activity in tumor initiation, we examine tumorigenesis in genetic models of Msi loss in vivo. First, we generated compound Msi1flox/flox;VillinCreER::APCmin/+ (Msi1−/−), Msi2flox/flox;VillinCreER::APCmin/+ (Msi2−/−), and Msi1flox/flox;Msi2flox/flox;VillinCreER::APCmin/+ (Msi-DKO) mice enabling individual or concomitant ablation of the two Msi genes throughout the epithelium by tamoxifen-mediated activation of a Villin-CreER allele (el Marjou et al., 2004; Figures S7A–S7C). Acute deletion of Msi1 in otherwise wild-type mice had no effect on Msi2 expression and vice versa (Figure S7D). To study the effects of Msi loss on tumorigenesis, Msi genes were deleted alone or concomitantly followed by maintaining mice on a low protein diet to promote adenoma formation upon loss of heterozygosity at the APC locus. Individual deletion of either Msi1 or Msi2 had no effect on tumor burden in the APC<sup>min/−</sup> background (Figure S7E). In contrast, Msi-DKO mice had a significant reduction in tumor burden relative to controls (Figure 7E). One hundred percent (68/68) of residual tumors forming in Msi-DKO mice were found to have escaped recombination at one or more of the floxed Msi alleles, and thus Msi−/− tumors were never observed (Figure 7F). These data strongly indicate that Msi proteins act redundantly as oncogenes, with the presence of either Msi1 or Msi2 being sufficient to support tumorigenesis upon APC loss.

Whereas the APC<sup>min/−</sup> model is relevant to human disease in that spontaneous loss of APC is found in the vast majority of human CRC (Kinzler et al., 1991; Miyoshi et al., 1992; Nagase et al., 1992), the mouse model differs from the human condition in that tumors are primarily localized to the small intestine rather than colon, and they rarely progress to malignant adenocarcinoma. We therefore examined tumor formation in the AOM-DSS model of inflammation-driven colorectal adenocarcinoma (De Robertis et al., 2011). This model is clinically relevant as chronic inflammation is a leading indicator of CRC risk (van Hogezaend et al., 2002). Experimental and control mice were given a single dose of the mutagen azoxymethane (AOM), followed by cycles of the inflammatory agent dextran sodium sulfate (DSS) to drive formation of colorectal adenocarcinomas (Figure S7F). As expected, colorectal adenocarcinomas reproducibly formed in control mice (Figures 7G–7I and S7G). In contrast, Msi-DKO mice were completely resistant to tumorigenesis in this model with zero tumors forming (Figures 7G–7I and S7G). Taken together, our findings demonstrate the Msi1 and Msi2 act as potent, redundant oncoproteins whose activity is required for the initiation and maintenance of cancers of the intestinal epithelium.

**DISCUSSION**

The Msi family of RNA-binding proteins has been implicated in oncogenic transformation in a number of organ systems, through...
Figure 5. Msi1 Functions Through the PDK-Akt-mTORC1 Axis

(A) CLIP-seq track showing Msi1 binding to the 3' UTR of the Pten tumor suppressor mRNA.

(B) Immunoblotting for Pten and S6 phosphorylation upon Msi1 induction in the intestinal epithelium.

(C) PTEN enzymatic activity measured by immunoprecipitation followed by ELISA upon knockdown of MSI1 in 293T cells (**p < 0.005; Student's t test).

(D) Immunoblot analysis of the PI3K-AKT-mTORC1 pathway downstream of Pten in the intestinal epithelium of two control and two TRE-Msi1 mice treated with Dox for 24 hr.

(E) Immunofluorescence for phosphorylation of S6 by the mTORC1 target S6 kinase in control and TRE-Msi1 transformed intestinal epithelium (scale = 200 μm).

(F–H) Rapamycin treatment rescues TRE-Msi1-induced transformation of the intestinal epithelium. Mice treated with rapamycin for 3 days prior to Dox administration exhibit decreased crypt fission (F) and a block in crypt height expansion (G; *p < 0.05; **p < 0.005; ***p < 0.0005; Student’s t test). (H) Immunofluorescence staining for Ki67 of Dox-induced TRE-Msi1 mice with or without rapamycin treatment (scale = 100 μm). Brackets indicate the height of the crypt proliferative zone.

See also Figure S5.
a number of proposed downstream mechanisms. In particular, several RNA-binding targets have been proposed to mediate the oncogenic function of Msi1, including the transcripts encoding p21, Numb, APC, and others (Battelli et al., 2006; Cambuli et al., 2015; Imai et al., 2001; Sanchez-Diaz et al., 2008; Spears and Neufeld, 2011; Sureban et al., 2008; Uren et al., 2015; Vo et al., 2012). These mechanistic studies, however, were conducted using transformed cell lines in vitro or constitutive, random integrant gain-of-function transgenesis. In contrast, in vivo transcriptome-wide RNA binding analyses for Msi1 and Msi2 suggest that the reality is far more complex, with Msi interacting with hundreds to thousands of transcripts.

Although published studies have focused largely on Msi1, the second mammalian Msi family member, Msi2 has recently emerged as a potent oncoprotein. In the hematopoietic system, Msi2 is the only Msi family member expressed, and its activity is required for hematopoietic stem cell self-renewal (Park et al., 2014). Further, oncogenic Msi2 activity promotes aggressive leukemias, in the context of acute myelogenous leukemia (AML), mixed-lineage leukemia (MLL), and chronic myelogenous leukemia (CML), where it drives chronic phase disease into the more-aggressive blast crisis phase characterized by a more stem-cell-like gene expression profile. Interestingly, in the hematopoietic system, the effects of Msi2 appear to be largely independent of the previously identified Msi1 RNA-binding targets encoding Numb and p21 (Kharas et al., 2010; Park et al., 2014, 2015). Rather, Msi2 appears to be working through the TGF-β, Hoxa9, Ikzf2, and Myc mRNAs, as well as through a more-general function in regulating RNA biogenesis in the hematopoietic lineage.

In the intestinal epithelium, Msi2 governs RNA biogenesis and also has potent oncogenic properties consistent with observations in the hematopoietic system (Wang et al., 2015). In contrast to the hematopoietic system, however, Msi2 bound to several transcripts encoding well-established colorectal tumor suppressors, including the negative regulator of ErbB-signaling Lng1, as well as Bmpr1a and Pten (Goel et al., 2004; He et al., 2004, 2007; Howe et al., 2001; Marsh et al., 2008; Naguib et al., 2011; Powell et al., 2012; Wang et al., 2015). Interestingly, the known Msi1-binding targets encoding p21 and Numb were found to bind Msi2 in vivo; however, Msi2 induction does not elicit a strong up-regulation of the Notch pathway, nor does Msi2 potentiate activity of the canonical Wnt pathway as proposed for Msi1 in cell culture models. Instead, the major oncogenic effects of Msi2 in vivo...
Figure 7. MSI1/Msi1 and MSI2/Msi2 Cooperate to Promote Tumor Growth In Vivo

(A and B) Growth of RKO cell xenografts upon MSI or β-CATENIN shRNA knockdown (A), with tumors shown after dissection upon termination of the experiment (B).

(C and D) Growth of RKO cell xenografts upon combined knockdown of MSI proteins alone or with β-CATENIN knockdown (C), with tumors shown after dissection upon termination of the experiment (D; **p < 0.005; ***p < 0.0005; Student’s t test).

(legend continued on next page)
appears to be activation of mTORC1, a complex dispensable for intestinal function but known to be required for intestinal tumorigenesis downstream of APC loss (Faller et al., 2015; Fujishita et al., 2008). Thus, there exist discrepancies between the proposed oncogenic functions of Msi1 in CRC cells (e.g., suppression of p21, Numb, and APC translation and potentiation of Wnt signaling) and the acute oncogenic consequences of Msi2 gain of function in vivo (RNA metabolism and mTORC1 activation). Whether these discrepancies represent actual biological differences between the activity of the two Msi family members or differences between the in vitro and in vivo model systems were not understood.

Here, we generated a single-copy, inducible Msi1 mouse model using genetic loci and strains identical to those previously used for Msi2 induction in vivo, enabling a direct comparison of the oncogenic consequences of acute gain of function of the two Msi family members. Remarkably, Msi1 induction elicits the identical phenotypic transformation of the intestinal epithelium observed for Msi2 through interaction with a largely common repertoire of transcripts. Interestingly, we observed that Msi1 did bind to the APC mRNA as reported in vitro; however, we observed a stronger interaction between Msi1 and the Ctnnb1 mRNA, suggesting that Msi1 might regulate the canonical Wnt pathway. However, in contrast to prior reports, we found no evidence that Msi1 potentiates Wnt pathway activity either in vitro or in vivo, using either human or mouse Msi1/Ms1, consistent with our observation that Msi1/2 knockdown in human CRC cells does not significantly affect levels of nuclear β-CATENIN. Further, the observation that β-CATENIN knockdown does not affect Msi1 levels supports a model in which the oncogenic activities of Msi1 and β-CATENIN lie in parallel pathways.

These data indicate that Msi1 and Msi2 function redundantly, leading to the prediction that Msi proteins can act interchangeably as oncogenes in CRC. Indeed, dual inhibition of both Msi proteins is required to fully abrogate tumor growth in CRC cell lines. Interestingly, the dependence of these cells on Msi activity appears to be independent of underlying genetic driver mutations, as Msi inhibition was effective in blocking growth of RKO cells (mutations in BRAF, PIK3CA, and microsatellite instability), SW48 cells (mutations in APC and microsatellite instability), HCT116 (mutations in KRAS, PIK3CA, TP53, β-CATENIN, and microsatellite instability), and LoVo cells (mutations in KRAS, APC, and microsatellite instability; Ahmed et al., 2013). This observation is similar to what was previously observed for Msi2 in human leukemias, where Msi2 upregulation and subsequent oncogene addiction occurs regardless of underlying genetic mutation (Kharas et al., 2010).

Further, despite the focus on Msi1 in CRC, our data suggest that Msi2 may be the dominant Msi family member in driving these malignancies, as Msi2 upregulation is essentially ubiquitous in CRC, whereas Msi1 upregulation is observed only in a subset of human tumor samples and CRC cell lines. Why Msi2 is preferentially expressed relative to Msi1 remains unclear.

Ultimately, the current study demonstrates that both Msi family members elicit an analogous transformation of the intestinal epithelium, with identical phenotypic changes, highly similar gene expression profiles, and overlapping RNA-binding targets. Further, concomitant loss of both Msi family members was sufficient to abrogate tumorigenesis in both human and murine models, clearly demonstrating the critical importance of these RNA-binding proteins to the ontogeny of CRCs. Our findings serve as a basis for exploring the mechanisms through which Msi proteins promote human cancers and highlight the critical importance of addressing the role of both family members when studying Msi function and consideration of their redundant function for development of Msi inhibitors.

**EXPERIMENTAL PROCEDURES**

**Generation of TRE-Msi1 Mice**

All procedures involving mice were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania (Animal Welfare Assurance reference number A3079-01; approved protocol no. 803415 granted to Dr. Lengner) and were in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals of the National Research Council of the NIH. Euthanasia was performed using controlled flow carbon dioxide administration followed by cervical dislocation.

The murine Msi1 cDNA (a kind gift from Dr. Joseph Verdi, Maine Medical Center Research Institute) was cloned into the unique EcoRI restriction site of the pBS31 vector containing a PGK promoter followed by an ATG start codon and an FRT recombination site, followed by a splice acceptor-double polyA cassette, the tetracycline operator with a minimal CMV promoter, the unique EcoRI site, and the SV40 polyadenylation signal. The pBS31-Msi1 vector was then electroporated along with a Flpe recombinase-expressing vector into KH2 embryonic stem cells harboring the modified reverse tetracycline transactivator (M2rtTA) targeted to and under transcriptional control of the ROSA26 locus, as well as an FRT-flanked PGK-neomycinR cassette followed by a promoterless, ATG-less hygromycinR cassette targeted downstream of the Collagen1a1 locus. Selection for hygromycin resistance upon flip-in yielded numerous colonies that were verified for proper recombination at the Coll1a1 locus by digestion of genomic DNA and Southern blotting with a 3′ internal probe, yielding a 6.2-kb wild-type band, a 6.7-kb band for the FRT-containing knockin allele, and a 4.1-kb band for the successfully flipped-in Msi1-inducible allele (TRE-Msi1).

**CLIP-Seq and CLIP-qRT-PCR**

CLIP-Seq libraries were made as previously described in Chi et al. (2009) with modifications. The CLIP-seq procedure used in this study was identical to that described for the TRE-Msi1 model. Click-assisted CLIP (CLIP-Seq) was performed using a TRE-Msi1 inducible allele with an ATG start codon followed by an FRT recombination site, FRT-flanked PGK-neomycinR cassette followed by a promoterless, ATG-less hygromycinR cassette targeted downstream of the Collagen1a1 locus. Selection for hygromycin resistance upon flip-in yielded numerous colonies that were verified for proper recombination at the Coll1a1 locus by digestion of genomic DNA and Southern blotting with a 3′ internal probe, yielding a 6.2-kb wild-type band, a 6.7-kb band for the FRT-containing knockin allele, and a 4.1-kb band for the successfully flipped-in Msi1-inducible allele (TRE-Msi1).

(E) Frequency of intestinal adenomas in APC<sup>min/+</sup> mice with or without deletion of Msi gene deletion in Msi1/2<sup>APC<sup>min/+</sup>:Villin-CreER</sup> mice (**p < 0.005; Student’s t test; n = 5-6 mice per group).
(F) Representative immunofluorescence micrographs of residual tumors in Msi1/2<sup>APC<sup>min/+</sup>:Villin-CreER</sup> mice showing Msi1 (red), Msi2 (green), or Msi1/2 expression (scale = 100 μm). The graph at right depicts number of residual tumors in Msi1/2<sup>APC<sup>min/+</sup>:Villin-CreER</sup> mice that were either positive or negative for Msi immunoreactivity (100% or 68/68 total residual tumors scored positive).
(G) Frequency of inflammation-driven colorectal adenomas/adenocarcinomas in mice treated with the AOM-DSS protocol, with or without prior Msi gene deletion in Msi1/2<sup>APC<sup>min/+</sup>:Villin-CreER</sup> mice (**p < 0.005; Student’s t test; n = 6 mice per group).
(H) Photographs of distal colon resected from control (left) and Msi1/2 double knockout (right) mice at the end of the AOM-DSS protocol.
(I) Representative H&E histological section of a colorectal adenocarcinoma resulting from AOM-DSS treatment in control mice (left). In contrast, mice lacking Msi gene function exhibited normal colon morphology after the AOM-DSS protocol (right; scale = 200 μm).

See also Figure S7.
used in Wang et al. (2015) and is described in detail in the Supplemental Experimental Procedures.

**Generation and Verification of Msi Floxed Alleles**

Msi1 and Msi2 alleles were targeted using homologous recombination in V6.5 embryonic stem cells. Generation of the Msi2 conditional allele is described in Park et al. (2014). Targeted (3-loc) clones were isolated after neomycin (G418) selection, and Southern blotting using external probes flanking both the 3’ and 5’ targeting arm validated proper insertion of the targeting vector. Three-loc clones were then transiently electroporated with Cre recombinase and subcloned to identify 2-loc conditional clones. Clones harboring 2-loc (floxed) Msi1 and Msi2 alleles were injected into blastocysts and resulting chimera backcrossed to a Black/6 background. Addition of Cre recombinase (either transiently in culture or through intercrossing with Villin-CreER mice) resulted in deletion of the transcriptional start site and exons 1 and 2 (in the case of Msi1) or exons 1–4 (in the case of Msi2), generating a 1-loc null allele that was validated by Southern blotting and Msi protein loss. Southern blotting was carried out by digesting genomic DNA overnight, transferring to Hybond XL membrane (Amersham/GE Healthcare) and hybridizing with a 32P-probe labeled by random priming (Prime-it II, Agilent Technologies) in Church buffer at 60°C overnight followed by washing with increasing stringency SDS/SSC buffer and exposure to film.

For Msi1/2 in vivo expression analysis in Figure S7D, Msi1flox/+;VillinCreER or Msi2flox/+;VillinCreER mice were administered five doses of tamoxifen (2 mg/dose in 100 μl corn oil) when they were 6 weeks old. After a 5-day chase period, crypt epithelial RNA was isolated and Msi gene expression was analyzed.

**Apcmin/+ and AOM-DSS Tumor Models**

The Apcmin/+ mice were obtained from JAX lab (stock number: 002020). Apcmin/+;Msi1flox/+;Msi2flox/+ or Apcmin/+;Msi1flox/+ mice (n = 6–7 mice per group) with or without the Villin-CreER allele (el Marjou et al., 2004) were given five doses of tamoxifen (2 mg/dose in 100 μl corn oil) when they were 6 weeks old and then raised on a low-protein/high-fat diet (Research Diets; D12079B) for 6 months. Additional series of tamoxifen doses was given 2 and 4 months post-initiation of high-fat diet to maximize recombination of floxed Msi alleles. Adenomas were scored by two independent researchers using a stereomicroscope for Apcmin/+ or scored histologically for Apcmin/+ and/or Apcmin/+:Msi1flox/+;Msi2flox/+ mice.

For AOM-DSS, control mice were co-housed with experimental mice and injected with 100 μl of 20 mg/ml tamoxifen every day for 5 days (n = 6 mice per group). Two weeks after the final tamoxifen injection, all mice were given a single intraperitoneal injection of 10 mg/kg 1 of AOM. One week later, all mice received 3% DSS in their drinking water for 7 days and then regular water for 14 days. Two more cycles of DSS were subsequently administered, with the third and final cycle reduced to 2.5% DSS. Fecal samples were collected regularly during the experiment, and mice were observed daily and weighed weekly. Mice were sacrificed approximately 5 weeks after the last DSS cycle. Tumors were counted using a stereomicroscope, and colon tissue was subsequently fixed for histology.

**ACCESSION NUMBERS**

The accession number for the transcriptome profiles reported in this paper is GEO: GSE54598. The accession number for the CLIP-seq data reported in this paper is GEO: GSE54398.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.11.022.

**AUTHOR CONTRIBUTIONS**

N.L., Z.Y., and C.J.L. designed all experiments, executed the majority of experiments, and wrote the manuscript. M.Y. performed histological and flow cytometric analysis. A.-N.-D. performed colorectal-tumor-modeling experiments. F.L., L.V., A.S.N., R.J.C., and B.D.G. performed analysis of sequencing data. K.P. and A.-N.-D. managed animal colonies and performed luciferase assays. D.-H.W. performed human patient sample expression analysis. S.W. performed histological analysis. S.R., J.T., and S.T.J. performed analysis of transcriptome profiling data. G.M. performed electrophoretic mobility shift assays. M.G.K. and G.J.L. generated TRE-Msi1, Msi1flmox and Msi2flmox mouse strains. T.S.B. performed husbandry with these strains, M.G.K. contributed to editing the manuscript. A.V. and P.S.K. contributed to the conception of mTorc1-related experiments and mTorc1 pathway analysis as well as reagents. R.P.D. contributed to the development and application of the in vivo CLIP-seq procedures. K.Y. performed TCGA data analysis. S.S. performed histological analysis and quantification.

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